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# Vesicular transport across the fungal cell wall

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**Recent findings indicate that fungi use vesicular transport to deliver substances across their cell walls. Fungal vesicles are similar to mammalian exosomes and could originate from cytoplasmic multivesicular bodies. Vesicular transport enables the export of large molecules across the cell wall, and vesicles contain lipids, proteins and polysaccharides, many of which are associated with virulence. Concentration of fungal products in vesicles could increase their efficiency in food acquisition and/or delivering potentially noxious substances to other cells, such as amoebae or phagocytes. The discovery of vesicular transport in fungi opens many new avenues for investigation in basic cell biology and pathogenesis.**

## A complex wall encases fungal cells

Among the eukaryotes, cell walls are found in many plant, fungal and algal species. The fungal cell wall is located outside the cell membrane and serves a protective function by providing rigidity, mechanical strength and shielding of the protoplast from the environment. Fungal cell walls are composed of a tight, semipermeable fibrillar network of polymers such as chitin, glucan polysaccharides and mannoproteins [1]. Most human pathogenic fungi have cell walls, which are important targets for antifungal drug discovery. The importance of the cell wall for fungal cell survival is evident by the fact that echinocandins-type antifungal drugs that disrupt cell wall biosynthesis in some species are fungicidal. The ability of the cell wall to protect the cell by limiting access to outside molecules also provides a potential barrier to diffusion of fungal products. Fungi in the environment obtain their food by digesting organic matter in their environment with enzymatic cocktails that produce small molecules that are then absorbed. Consequently, fungal cells must have efficient mechanisms for the transport and export of cellular products required for nutrient acquisition.

## The porosity of the cell wall

Analysis of isolated cell walls indicates that they are semipermeable structures with a limited and defined permeability. The porosity of fungal cell walls vary with the study and the method used. Early studies of *Neurospora* cell wall permeability by exclusion methods reported a threshold of only 5 kDa [2], a size incompatible with the secretion of many proteins. However, later studies indicated that the fungal cell wall was permeable to much larger molecules [3]. The observation that fungal products often exceeded the exclusion size measured for cell wall permeability was recognized as an important question in the field for some time [3]. For *Cryptococcus neoformans*, the exclusion threshold to dextrans, was 30.6 and 270 kDa for melanized and non-melanized cells, respectively [4]. An alternative to trans-cell wall diffusion was suggested with the atomic force microscopy observation that *Saccharomyces cerevisiae* cell wall had pores of around 200 nm that could increase to 400 nm in stress conditions [5]. However, such putative pores remain poorly characterized and their physiological function remains poorly understood. Additional evidence for the existence of pores on fungal cell walls comes from cryoporometry studies on acid-resistant melanized cell walls of *C. neoformans* by cryoporometry, which revealed a population of pore sizes ranging from 1–4 nm to 30 nm [6]. However, cryoporometry could not establish whether the pores spanned the cell wall or simply existed as spaces within that structure. Consistent with observations from dextran permeability studies [4], the apparent pore size of cryptococcal cells measured by cryoporometry was reduced by progressive cell wall melanization [6]. Notably, the pore sizes of melanized cells could be blocked by monoclonal antibodies to melanin, indicating that the pores were distributed on the cell surface [6]. In evaluating studies of cell wall permeability is it worthwhile to consider that these methods employ isolated cell walls recovered by harsh methods such as alkaline and acid extraction that could damage the cell walls and overestimate measured pore sizes. From these studies, one might infer that the cell wall is freely permeable to small molecules

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such as simple carbohydrates and amino acids but presents a diffusion barrier for larger molecules. Furthermore, melanization reduces pore size and melanotic fungal cells can be assumed to have reduced cell wall permeability.

### The problem posed by *C. neoformans*

Cryptococcal spp. are distinctive in that they have a polysaccharide capsule. Among cryptococcal spp., the human pathogenic yeast *C. neoformans* has been studied extensively. *C. neoformans* has a large polysaccharide capsule that is an essential virulence factor. Furthermore, *C. neoformans* sheds copious amounts of polysaccharide into extracellular spaces and can form highly tenacious biofilms in which cryptococcal cells are enmeshed in a polysaccharide matrix [7]. Analysis of the capsular polysaccharide and exopolysaccharide reveals that this material is composed of macromolecules with molecular mass in the range of 0.5–7 MDa [8]. This material is organized into a capsule that can acquire gargantuan dimensions, reaching diameters up to 50 μm. The observations that the capsular polysaccharide is synthesized in cytoplasmic vesicular structures [9–11], that capsular polysaccharide has a mass 0.5–7 MDa [8] and a cryptococcal cell walls have an exclusion size of 270 kDa [4] in combination pose the question of how these macromolecules are exported across the cell wall. Although this problem could be circumvented by intracellular synthesis of smaller precursor molecules that diffuse across the cell wall for assembly into macromolecules in the extracellular space, as has been described for cell wall glycans [12,13], chitin [14] and 1,3-β-glucan [15], this problem nonetheless suggested to us the vesicular transport hypothesis. Our thinking in this matter was influenced by the observation that several monoclonal antibodies (mAbs) to capsular polysaccharide bind to intracellular material that is assembled on Golgi-derived vesicles [11]. Because some of these mAbs bind to complex conformational epitopes [16] on the polysaccharide, we interpreted this result as indicative that the capsular material underwent advanced assembly before extracellular export. Fully cognizant that other viable explanations for macromolecular assembly in the extracellular space existed, the hunch that trans-cell wall vesicular transport was involved in capsular assembly was pursued. The fact that the experimental search for vesicles in culture supernatants was technically easier than validating or refuting other explanations for the mechanism of capsule formation also influenced the approach and catalyzed the discovery of a trans-cell wall vesicular transport system in fungi [17]. Although polysaccharide-containing vesicles were easily found in *C. neoformans* [17] culture supernatants, numerous experiments were done to rule out artifacts and their reality is supported by several lines of evidence (Box 1).

### Early hints for vesicular transport

Although the notion that fungi use vesicles to transport material across the cell wall is novel, the idea that vesicles transport material to the extracellular space between the cell membrane and cell wall is well established. Vesicular transport is associated with the growth of hyphal tips and septae. For *C. neoformans*, an early association between vesicles and capsule synthesis came from the observation that metabolically active cells manifesting capsule enlargement had numerous vesicular-like structures in the cell wall [18,19]. Later, glycosphingolipid glucosylceramide, a lipid constituent of vesicles that migrate from the cytoplasm to the cell wall, was found in the cryptococcal cell wall consistent with the presence of vesicles in cell wall fractions [20]. Interestingly, the observation that human antibodies to glycosphingolipid glucosylceramide inhibited fungal growth, necessarily posited the presence of lipid on the external surfaces of the cell wall given that immunoglobulins would not be expected to penetrate the cell wall [20]. Similarly, antibodies to melanin were fungistatic for *C. neoformans* [21] and *Fonsecae pedrosoi* [22], possibly by mechanism such as pore blocking [6].

**Box 1. The reality of extracellular vesicles**

From the time of their initial observation in 2005, there has been tremendous effort to rigorously ascertain the reality of vesicles and rule out their existence as an artifact of culture. This effort is particularly important given that lipids have a propensity for self-assembly as evident from the ease in which liposomes and other vesicular structures can be generated from simple lipid preparations. The arguments for the reality of extracellular vesicles are based on the following observations: (i) No vesicles were recovered from fungal cell cultures killed by two different methods, including heat and azide [17]. (ii) Vesicles were visualized by electron microscopy traversing the cell wall in *C. neoformans* and *H. capsulatum* and in the capsule region of *C. neoformans* [17]. (iii) Vesicles come in discrete populations of certain dimensions and electron microscopic appearance, a finding in contradistinction to the heterogeneity and randomness that would be expected from lipid self-assembly [27]. (iv) Cytoplasmic and plasma membrane-associated multivesicular bodies containing vesicles of similar dimensions to those found in the extracellular space indicate that these structures are akin to mammalian exosomes [27]. (v) Extracellular vesicles contain numerous enzymatic activities such as laccase and urease and dozens of other proteins, an association that argues against random and spontaneous synthesis [26,27]. (vi) Metabolic labeling studies revealed that only viable cells assembled lipids into vesicles [27]. (vii) Electron microscopy revealed accumulation of vesicles in the cell wall during periods of *C. neoformans* capsular growth consistent with vesicle-associated vesicular transport to the extracellular space [18,19]. (viii) Specific mutations to a secretory pathway lead to the ablation of extracellular vesicles in *C. neoformans* [33].

### Traversing the cell wall

The finding of extracellular vesicles immediately poses a new problem: how do vesicles get across the cell wall? Electron microscopy has shown vesicle-like structures in the fungal cell wall [17], implying that vesicles can inhabit cell wall spaces, at least transiently. Because vesicles recovered from fungal culture supernatants have diameters that range from 60–300 nm, the claim that fungi export materials by vesicular transport begs the question of how such large vesicles traverse the cell wall. As noted earlier, a potential solution to this problem comes from the report that the fungal cell wall contains pores of 200–400 nm [5], with the important caveat that the existence of channels of such dimensions have not been proven. Given that the vesicles are composed of lipid bilayers, the vesicles are likely to be compressible to diameters smaller than when they are in an ovoid form. Even assuming the existence of suitably sized channels, there remains the problem of how a vesicle is transported through those

apertures. In this regard, one might imagine that a molecular motor might be needed for efficient vesicular transport across the cell wall. In is regard, it is noteworthy that actin and myosin are involved in numerous cell wall processes including septa formation, hyphal growth, germination and sporulation [23–25], and it is conceivable that comparable motors exists to shuffle vesicles from the periplasmic space to the cellular exterior. Any speculation about the existence of cell wall molecular motors must also contend with the need for energy sources to power such transport machinery. Given that vesicles contain lipids, proteins and polysaccharides [17,26,27], and that many of the proteins identified in vesicle fractions are involved in energy metabolism, perhaps the vesicles contain the energetic molecules needed to power such motors.

## Vesicles are made by both ascomycetes and basidiomycetes

The trans-cell-wall vesicular transport system was described first for *C. neoformans* [17], and this immediately raised the question of whether this was a cryptococcal-specific transport mechanism or a general feature of fungal cells. Given that encapsulated yeast species are relatively rare and that *C. neoformans* is a basidiomycetes, was this phenomenon extended to the ascomycetes? A search for vesicles in the culture supernatants of *S. cerevisiae*, *Candida albicans*, *Candida parapsilosis*, *Histoplasma capsulatum* and *Sporothrix schenckii* revealed similar structures to those described for *C. neoformans* (Figure 1) [26]. The finding of vesicles among such phylogenetically distant organisms strongly indicates that trans-membrane vesicular transport is a general export solution among fungi.

## Vesicular cargo and origin

Analysis of vesicle cargo from fungi reveals that they contain proteins, lipids and, in some cases, polysaccharides [17,26,27]. Proteomic analysis reveals a surprisingly complex protein composition including enzymes, biosynthetic and structural proteins, which includes several components associated with virulence. Electron microscopic examination of vesicles isolated from culture supernatants revealed various morphologies including electron dense contents consistent with the notion that different vesicles carry different types of cargo [17,26,27]. This, in turn, indicates that vesicles could be classified depending on their cargo and implies the existence of different vesicle assembly pathways that could be identifiable by genetic analysis that would correlate specific mutations with the deletion of specific cargo components and/or vesicle populations (Figure 2).

The identification of vesicular protein components combined with electron microscopic analysis of fungal cells showing vesicles within larger membrane-bounded structures has led to the proposal that the extracellular vesicles are structures akin to mammalian exosomes [27]. Exosomes are vesicular structures of 30–100 nm that are produced by mammalian cells as intraluminal vesicles inside cytoplasmic multivesicular bodies [28,29]. In mammalian cells, exosomes are involved in cell-to-cell communication and have been implicated in such diverse roles as antigen presentation, tumor metastasis and viral spread [29]. The proposal that the extracellular vesicles found in fungal cultures are comparable structures to mammalian exosomes was based on similarities in cargo and morphology, and the observation of multivesicular structures on the fungal cytoplasm [27]. However, even if vesicular synthesis in fungi and mammals is phylogenetically related, it is likely that such vesicles would have different functions given their very different biology.

## The advantages of concentration and 'virulence factor bags'

The finding of numerous enzymes and components associated with virulence in vesicles raises the question of what advantages this could have for fungal cell nutrition and survival. Fungal cells are classically viewed as obtaining all their nutrition by digesting organic matter in their environment. Consequently, fungi produce powerful enzyme cocktails that degrade plant matter, freeing components such as sugars, amino acids and lipids that are then metabolized for nutrition. However, plant matter is notoriously difficult to degrade and often requires complex

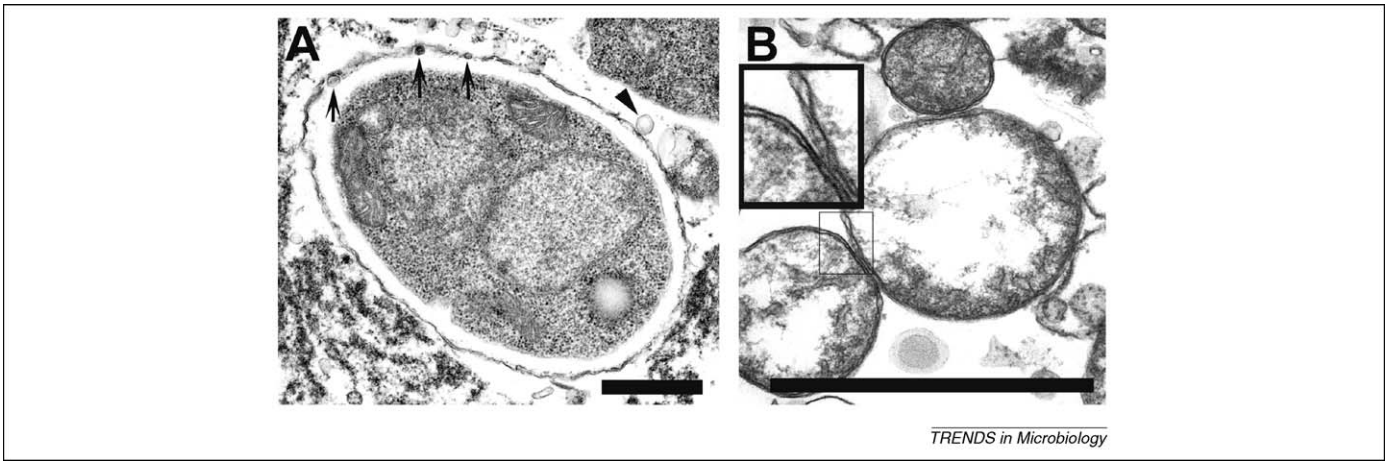


**Figure 1**. Vesicles in the cell wall of *Histoplasma capsulatum*. Electron microscopic image of **(A)** *H. capsulatum* yeast cell with vesicles within the cell wall (arrows) and associated with the cell wall (arrowhead). **(B)** Heterogeneous extracellular vesicles from *H. capsulatum* isolated by ultracentrifugation from fungal culture supernatants. Inset shows enlarged section of vesicle highlighting double layer membranes. Note the heterogeneity in size and electron dense appearance. Scale bars: (a) 0.5 μm; (b) 0.5 μm.

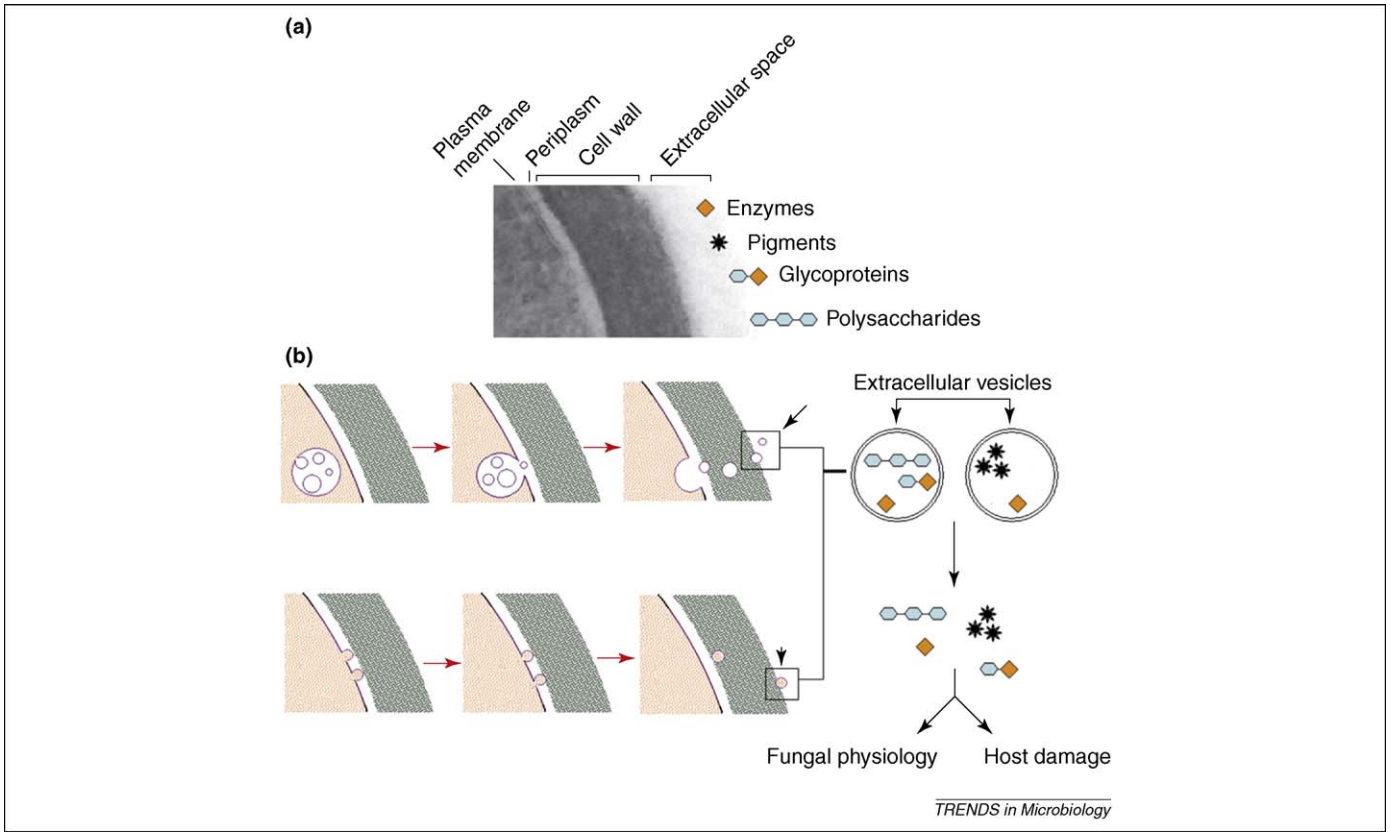


**Figure 2**. Scheme for vesicle synthesis in fungal cells. **(a)** The fungal cell wall. The production of extracellular enzymes, pigments, glycoproteins and polysaccharides by fungal cells implies the existence of trans-cell wall mechanisms of molecular export. **(b)** Mechanisms of vesicle formation and passage could involve multivesicular body formation for exosome release (upper panels) or simple membrane budding (bottom panels). Extracellular vesicles are labeled with arrows. Vesicle contents could be released by membrane lysis for use in regular physiologic events or for delivery into host tissues.

enzyme mixtures for breakdown. For example, white-rot fungi degrade lignin by secreting a cocktail of enzymes that include laccase, lignin peroxidases and manganese peroxidases [30]. Because the degradation of plant material presumably requires a complex set of enzymatic reactions occurring in a localized area, enzyme packaging in vesicles would insure simultaneous delivery of the enzymes to a relatively small area. Vesicular delivery would avoid the problem that enzymes released at the cell surface are rapidly diluted as they diffuse away from fungal cells. In this regard, it is noteworthy that wood degrading enzymes have been shown to be associated with membranous structures in some lignolytic fungi [31]. Packaging of enzymes in vesicles could provide fungi with the advantage of a higher digestive enzyme concentration for nutrient acquisition. In the case of mammalian virulence, vesicular packaging might also have a role in fungal cell defense, as the vesicles of *C. neoformans* and *H. capsulatum* concentrate several antioxidant proteins [17,26,27]. The combination of numerous protein, lipid and polysaccharide components associated with virulence [17,26,27] also implies that delivery of such components to host cells in a concentrated fashion could be far more damaging than if these are encountered by diffusion from fungal cells. Furthermore, it is possible that vesicular packaging enables more efficient uptake of fungal products by a target cell because of vesicle-host cell membrane fusion and/or vesicular uptake by phagocytic mechanisms. Both *C. neoformans* and *H. capsulatum* are soil organisms that are vulnerable to predation by protozoa, and many virulence factors for mammals have been demonstrated to also protect against amoeba [32]. Hence, it is conceivable that vesicles also serve to deliver noxious compounds to potential soil predators and thus have a role in fungal cell defense.

## Concluding remarks and future directions

The finding of extracellular vesicles that presumably function as delivery vehicles for transporting and concentrating large molecular weight cargo across the cell wall indicates towards new areas of investigation. The preliminary studies have allowed the notion of trans-wall cellular transport in fungi to progress from hypothesis to theory, with the full acknowledgement that additional data are needed before such a mechanism can be established as a fact. Nevertheless, the available data indicate a new hypothesis amenable to experimental testing. For example, the heterogeneity of vesicles could imply the existence of sophisticated mechanisms for vesicle generation and content insertion. Furthermore, the apparent heterogeneity in content indicate that there must exist an address and delivery system such that vesicles destined for delivery to the cell wall and immediate extracellular sites are handled differently than those whose function is to export enzymes for food acquisition. In this regard, one might think of system analogous to postal codes whereby vesicles are marked for far or near extracellular delivery and for cell wall delivery. Perhaps most daunting is the notion that relatively large vesicles can easily traverse the tough and

dense fibrillar network that is the fungal cell wall, a problem that invites such thoughts as pore formation and the possibility of extracellular motors. Clearly, just accepting the reality of extracellular vesicles in fungi poses numerous questions that require new thinking on cell wall structure and function.

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